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Mutation analysis of androgen receptor gene: Multiple uses for a single test

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ABSTRACT

Androgen receptor gene mutations are one of the leading causes of disorders of sex development (DSD) exhibited by sexual ambiguity or sex reversal. In this study, 2 families with patients whom diagnosed clinically as androgen insensitivity syndrome (AIS) were physically and genetically examined. This evaluation carried out by cytogenetic and molecular analysis including karyotype and sequencing of SRY and AR genes. In family 1, two brothers and their mother were hemizygous and heterozygous respectively for c.2522G > A variant, while one of their healthy brother was a completely normal hemizygote. Family 2 assessment demonstrated the c.639G > A (rs6152) mutation in two siblings who were reared as girls. The SRY gene was intact in all of the study's participants.
Our findings in family 1 could be a further proof for the pathogenicity of the c.2522G > A variant. Given the importance of AR mutations in development of problems such as sex assignment in AIS patients, definitive diagnosis and phenotype–genotype correlation could be achieved by molecular genetic tests that in turn could have promising impacts in clinical management and also in prenatal diagnosis of prospect offspring.
In this regard, phenotype–genotype correlation could be helpful and achieved by molecular genetic tests. This could influence the clinical management of the patients as well as prenatal diagnosis for the prospective offspring.

1. Introduction

Mutations in AR gene may lead to perturbation in the uptaking of androgen and subsequent Androgen Insensitivity Syndrome (AIS) by the effect of insufficient intracellular androgenic response. There are various AIS phenotype patients' varieties i.e. from isolated infertility in less severe type of Partial Androgen Insensitivity Syndrome (PAIS) to Complete Androgen Insensitivity Syndrome (CAIS) manifested by female phenotype. The prevalence of CAIS is about 1 in 40,000–60,000 birth (Brinkmann, 2001). All AIS patients regardless of phenotype severity show 46 XY karyotype, and the absence of Mullerian derived structures including fallopian tubes, uterus and cervix with normal testicular pathology found beyond physical, paraclinical and cytogenetic examinations. Based on the Quigley et al. outline, there are 1–5 degrees of AIS in which the first degree corresponds CAIS and the 2–5 degrees are in relation with the wide diversity in clinical presentations of PAIS. CAIS is diagnosed based on the complete female appearance and breast development during puberty along with undescendent testes with or without epididymis and vas deferens. PAIS grade 1 (degree 2 of AIS) could predominantly be considered in female cases and is easily distinguished from CAIS by the presence of clitoromegaly or labial fusion. In the third degree of PAIS however, in addition to gynecomastia, clitoromegaly develops more into a penis with the length of less than

Abbreviations: AF, Activation function; AIS, Androgen insensitivity syndrome; AR, Androgen receptor; CAIS, Complete androgen insensitivity; DHT, Dihydrotestosterone; DNA, Deoxy nucleic acid; DSD, Disorders of sex development; EDTA, Ethylene diamine tetra acetic acid; FSH, Follicular stimulating hormone; LBD, Ligand binding domain; LH, Luteinizing hormone; NTD, N terminal domain; PAIS, Partial androgen insensitivity; PCOS, Polycystic ovarian syndrome; PCR, Polymerase chain reaction; PGD, Pre-implantation genetic diagnosis; POF, Premature ovarian failure; PND, Pre-natal diagnosis; SRY, Sex region on Y (chromosome); DHEA-S, Dehydroepiandrosterone sulfate; A2, Androstenedione.

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1 cm called microphallus and the testes may descend into more mature labia majora recognized as bifid scrotum. AIS patients are predominantly male in 3rd grade of PAIS phenotype and exhibit hypospadias with normal or small size of penis, descendent or undescendent testes and gynecomastia. The last but least severe type of PAIS [mild AIS (MAIS)] patients may solely indicate spermatogenesis defects and gynecomastia with mild impaired virilization (Quigley et al., 1995). Although genotype–phenotype correlation is not strong in AIS, this clinical heterogeneity is in contingency with the domain of protein that has been mutated (Balducci et al., 1996; Chu et al., 2002) and finding this correlation would help to better diagnose AIS patients. Contrary to CAIS, in molecular analysis of more than 50% PAIS patients a distinct AR gene mutation was not found (Ahmed et al., 2000; Morel et al., 2002). However, it seems AR mutation evaluation is preferable to other diagnostic tests like IHC in the evaluation of individuals with AIS phenotype because of its simplicity in sample preparation. In that AIS phenotype is apparent which is highly significant in AIS pathogenesis. Biochemical and paraclinical tests including pelvic ultra sound and genetic analysis complete the pyramid in AIS diagnosis.

Androgen receptor (AR) protein that is encoded by AR gene is located on chromosome Xq11–12 that contains 8 exons. It is the fourth member of group C classification belonged to the sub-classification of 3 nuclear steroid receptors super family and is involved in the development and differentiation of male sexual characteristics (McPhaul et al., 1991). More than 500 various types of mutations have been reported in AR gene, which is the leading cause of male sex derivation in the spectrum of diseases called disorders of sex development (DSD). Among all of the putative mutations, point mutations comprise the most common type of alterations in pathologic conditions related to AR gene (Quigley et al., 1995).

Sex dissatisfaction and other psychologically related problems in these patients demand the investigation of AR gene mutation and its role in this issue. Although the reports are controversial in both sexes.

2. Patients and methods

2.1. Patients

This study was carried out on two Iranian families affected by AIS diagnosed on the basis of clinical presentations, biochemical and screening discoveries. The informed consent forms were filled up by every family member. Progesterone, 17-OH progesterone, DHEA-S, androstenedione (A2), testosterone (T), dihydrotestosterone (DHT) and estradiol were measured at 08:00 am in patients whose FSH and LH levels were high and subsequently after hCG test in case the patients were prepubertal. The ratios of T/DHT and T/A2 were calculated. The ratios of T/DHT > 20 and T/A2 < 0.8 were considered as 5α reductase and 17β-hydroxysteroid dehydrogenase type 3 deficiency, respectively (Dattani et al., 2011).

2.2. DNA extraction and karyotype

Six milliliters of whole blood was taken from every family member and 4 mL of it was transferred into tubes containing 200 μL EDTA for DNA isolation. The remaining 2 mL of whole blood was preserved in tubes containing heparin to be used for karyotype examination. DNA was isolated from peripheral blood of all samples by standard salting out method. The quality and concentration of the genomic DNA were determined through PCR products in (1.5%) gel electrophoresis and spectrophotometer respectively. The karyotype of all the patients enrolled was determined through cell culture and subsequent preparation of chromosome ideogram according to the common method of G-banding. It is comprised of cell culturing, harvesting and slide preparation. The slides were GTG banded and examined under light microscope and 20 chromosome spreads were examined.

2.3. Polymerase chain reaction (PCR) and sequencing

The exons 1–8 of AR gene residing on chromosome Xq12 were amplified using 10 primer pairs designed via Primer 3 software (http://frodo.wi.mit.edu/primer) (Table 1). PCR reaction was incurred in a 25 μL reaction mixture including 50 ng of each genomic DNA template. The mixture consisted of 20 mmol/L Tris–HCl pH 8.4, 50 mmol/L KCl, 1 mmol/L MgCl2, 10 pmol for each primer, 0.2 mmol/L for each dNTP mixture and also 1.25 units of Smart Taq DNA polymerase Fermentas, (Canada). After initial denaturation at 94 °C for 5 min, 37 PCR cycles was performed using Biorad thermocycler (Bio-Rad Laboratories, Hercules, CA, USA); each cycle included denaturation at 95 °C in 30 s, annealing at 60 °C in 30 s, extension at 72 °C for 30 s and final extension at 72 °C in 5 min. However, the mentioned annealing temperature was changed to 55 °C, 58 °C and 63 °C for exons 3, 4 and 7 respectively. The single exon of SRY gene on chromosome Yp11.3 was amplified using one pair of forward and reverse SRY specific primer for sequence analysis (Table 2). Each 25 μL PCR reaction contained 20 mmol/L Tris–HCl pH 8.4, 50 mmol/L KCl, 2 mmol/L MgCl2, 10 pmol of each primers, 0.2 mmol/L of each dNTP mix, 1.25 units of Smart Taq DNA polymerase (Fermentas) and 50 ng of genomic DNA. Amplification was also carried out on using thermal cycler (Corbet, USA) with an initial 7-minute denaturation at 96 °C, followed by 35 PCR amplification cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 60 s, elongation at 72 °C for 45 s, and extension at 72 °C for 10 min respectively. PCR products were subjected to electrophoresis on agarose gel (1.5%) followed by staining with ethidium bromide (1 μg/mL) to verify anticipated length of amplified fragments. Direct sequencing was used with primers (Table 2) to observe AR and SRY gene sequences alterations in each patient. Sequencing was carried out by MACROGEN company in South Korea using classic Sanger method with ABI. The analyzing of raw data was performed by Gene Marker software.

2.4. Immunohistochemistry (IHC)

The genital skin biopsies of IV1 and IV2 for the first family were embedded in paraffin. IHC for AR protein was performed on 5 μm sections using Pharm Dx immunohistochemistry kit (Dako, Cambridge, UK).

3. Results

Genitalia phenotype in siblings (two brothers) for the first family indicated microphallus, hypospadiasis, bilateral cryptorchidism and gynecomastia. Additional examinations by ultrasound also manifested

Table 1
Primer list used fro AR gene mutation detection.

Primer	Sequence
Ex1a-F	5′-AGG GAA GTA GGT GGA AGA TTC AG-3′
Ex1a-R	5′-CCT CGC TCA GGA TGT CTT TAA G-3′
Ex1b-F	5′-CCC CAC TTT CCC CGG CTT AAG-3′
Ex1b-R	5′-GCA CTG GAC GAG GCA GCT GCG-3′
Ex1c-F	5′-TCC GGG ACA CTT GAA CTG CCG-3′
Ex1c-R	5′-TGC CCT GGG CCG AAA GGC G-3′
Ex2-F	5′-AAT GCT GAA GAC CTG AGA CT-3′
Ex2-R	5′-AAA ATC CTG GGC CCT GAA AG-3′
Ex3-F	5′-CTA GAA ATA CCC GAA GAA AG-3′
Ex3-R	5′- GAG AGA CTA GAA AAT GAG GG-3′
Ex4-F	5′-GTG ATT TTC TTA GCT AGG GC-3′
Ex4-R	5′-ATC CCC CTT ATC TCA TGC TC-3′
Ex5-F	5′-GAC TGA CCA CTG CCT CTG CC-3′
Ex5-R	5′-TCA CCC CAT CAC CAT CAC CA-3′
Ex6-F	5′-TGT AAA CTT CCC CTC ATT C-3′
Ex6-R	5′-TAA TGG CAA AAG TGG TCC TC-3′
Ex7-F	5′-TGT GGT CAG AAA ACT TGG TG-3′
Ex7-R	5′-CTC TAT CAG GCT GTT CTC CC-3′
Ex8-F	5′-GCC ACC TCC TTG TCA ACC CT-3′
Ex8-R	5′-AGA GGA GTA GTG CAG AGT TA-3′

Table 2
Primer list used for SRY gene mutation detection.

Sequence	Primer
5′-CATGAACGCATTCATCGTGTGGTC-3′	*SRY1-F*
5′-AGAATTGCAGTTTGCTTCCCGCAG-3′	*SRY1-R*
5′-TTT CAA TTT TGT CGC ACT CTC C −3′	*SRY2-F*
5′-AAA GTG AGG GCT GTA AGT TAT C-3′	*SRY2-R*

inguinal undescendent testes and very small prostate gland, diagnosed as grade III PAIS. Genitalia phenotype of siblings (two sisters) manifested completely female and consequently diagnosed as CAIS. Further evaluations using ultrasound demonstrated two inguinal undescendent testes which had been removed by surgery as a prophylaxis of gonadoblastoma. The results of cytogenetic study featured 46, XY karyotype for patients signifying the male chromosomal sex. The ratios of T/DHT and T/A2 were normal and the level of precursors of testosterone was not high either after hCG test or after puberty and the level of testosterone was normal in all patients. The single exon of SRY gene was intact and free of either mutation or polymorphism in all examinations. In family 1 (Fig. 1), both patients were hemizygote for c.2522G > A variant which is responsible for the replacement of arginine residue by histidine. Genotyping of their normal brother confirmed his normal phenotypes, albeit their mother was identified as heterozygote for the same alteration (c.2522G > A). In family 2 (Fig. 2), molecular analysis revealed c.639G > A variant, hemizygote state in both affected assigned sisters. Their mother was a homozygote for this variant in which the glutamic acid amino acid remained unchanged. The IHC test of the genital skin cells showed weak positive results also detected in IV1 and IV2 members of family 1 (Fig. 3).

4. Discussion

Sex dissatisfaction and other psychologically related issues have significant role in sex changes determination such as AIS. Various studies have shown controversial results in sex satisfaction whether in respect to males or females.

According to Diamond et al., CAH patients who underwent feminizing genital surgery have demonstrated poor sexual function and satisfaction. Sexual dissatisfaction was evident in 25% of DSDs including PAIS whom were reared as male or female (Migeon et al., 2002a). Mazur et al. have demonstrated that some of the patients with PAIS had the same dissatisfaction regardless of their sex has been changed to either male or female (Mazur, 2005). However, in present study, the medical interventions in order to help gender development toward male in affected cases of family 1 have failed to bring sex fulfillment.

IHC and genetic molecular tests are being used for PAIS diagnosis. Considering the invasiveness of sample providing for IHC technique genetic molecular test is preferred since as well as not being invasive, it is more informative in comparison. Therefore, the genetic molecular test is preferred as the sampling is not invasive and the results are more informative compared to the IHC test.

IHC result of two PAIS patients in family 1 was weakly positive, which to some extent explains the partial phenotypes of these patients, suggesting that the aberrant AR protein still could stimulate the androgenic response. In the next step, we identified the 2225G > A (R841H or rs9332969) variant detected within helice 9 LBD domain of AR protein encoded by exon 7 in the two brothers with the similar phenotype compatible for PAIS manifestation. The outcome was the replacement of arginine by histidine in residue 841. Also, to examine the pathogenicity of this variant, DNA analysis and sequencing were performed on one of the normal siblings of this family and his mother for AR mutations. It has been reported that the arginine residue of 841 together with 774, 855, and 866 residues constitutes four hot spots in AR gene, which accounts for 25% of all missense mutations residing in AR gene (Wilson et al., 1974; Maes et al., 1980; Batch et al., 1993). Further, R841H mutation influences the binding and interaction of AR protein in its receptor (Lundberg Giwercman et al., 1998). Accordingly, LBD domain mutations were detected in a patient with genital ambiguity with very low androgen binding capacity (De Bellis et al., 1994), that further can indirectly lead to a decrease in AR mediated transcription activity of target genes through impairing the ligand binding activity and increase AR protein recycling (Omrani and S. G. S., 2006).

Based on this and similar studies, it can be suggested that R841H exchange is often in relation with PAIS, not CAIS, which means that the substitution is insufficient for the total AR function disruption. Therefore, it seems that R841H mutation which was observed within the helice 9 of LBD domain of AR protein is in correlation with PAIS phenotype.

In this regard, the diagnosis of every patient with genital ambiguity through AR gene examinations especially LBD domain at birth, could pave the way for early detections and more practical management. By the assessment of different types of DSDs in more than 90 cases, Migeon et al. recommended that thedetection of mutations within the AR gene can be the main basis of PAIS diagnosis (Migeon et al., 2002b). Therefore, the early diagnosis of PAIS by molecular genetic tests during the first few weeks of life assists in better decision making and sexual identity cares.

However, in family 2 (sibling sisters and mother), G639A exchange was present. G639A variation resides within the AF2 segment of NTD

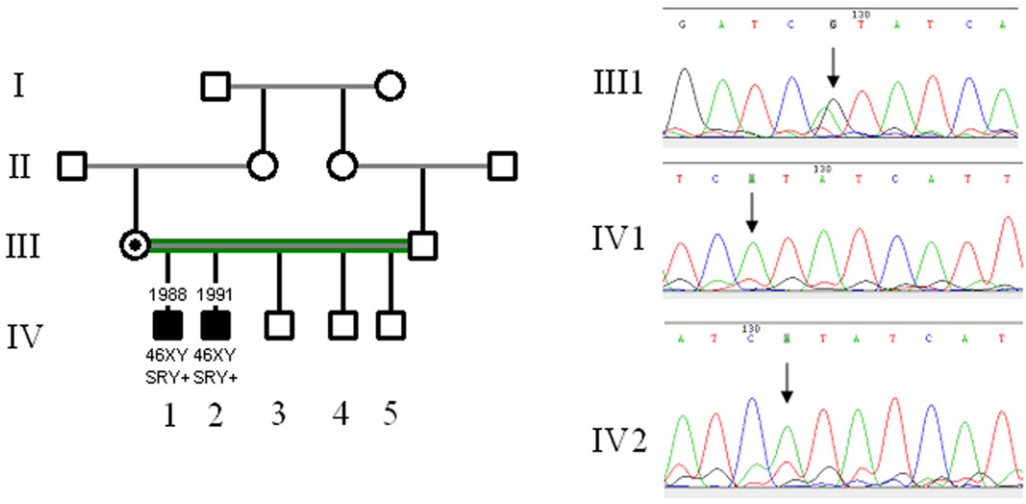

Fig. 1. Left: Family 1 pedigree; right: DNA sequence chromatograms in exon 7 of the AR gene for the affected sibling brothers; one normal sibling and their mother indicating the carrier statue of mother and the homozygote genotype of her two sons.

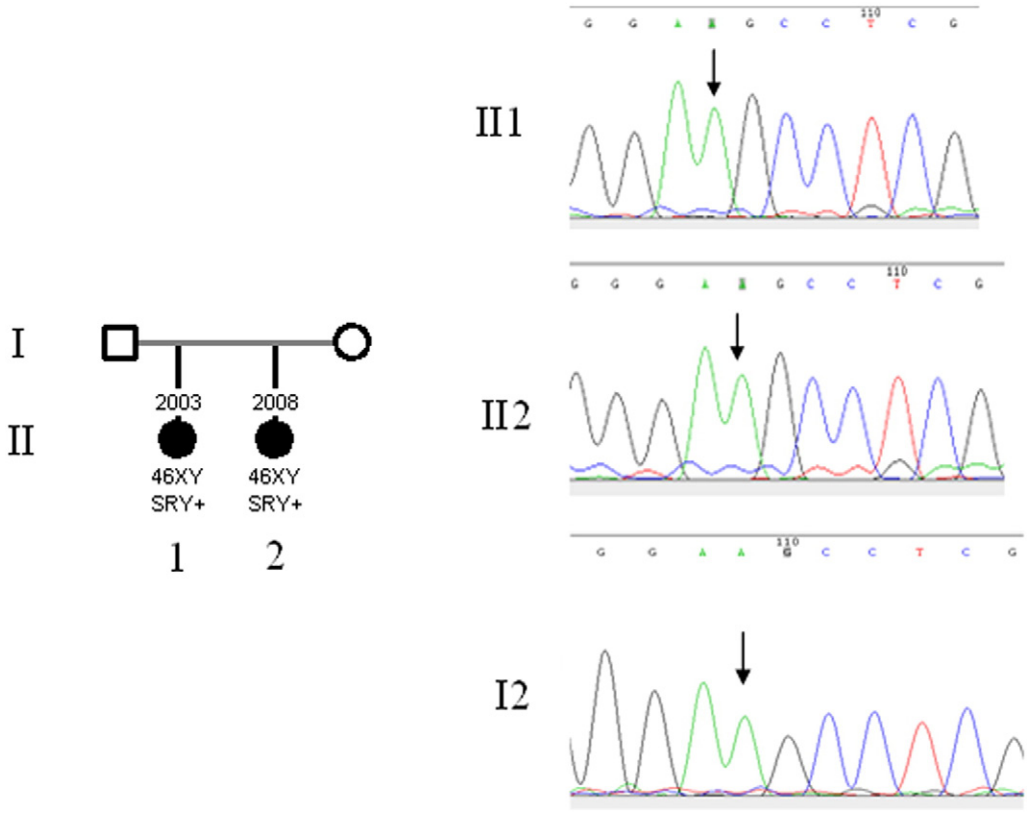

Fig. 2. Left: Family 2 pedigree; right: DNA sequence chromatograms of exon 1 of the AR gene related to the two affected sisters and their mother demonstrating the homozygote statue for mother and her actual two daughters.

which is encoded by exon 1 and is involved in the ligand activation transcription activity of AR. This synonymous and probably non-pathogenic variant was described before in a Brazilian family with five AIS patients (Melo et al., 2003). However, it has been reported in association with a related but not such close disease with AIS such as polycystic ovary syndrome (Lundberg Giwercman et al., 1998). Detecting the same finding in sequence analysis of the mother of family 2 (Fig. 2) which clarified that this polymorphism might have no effect on clinical presentations of her two sons. In addition, AR protein NTD domain has strong ability to be compatible with most of the single base pair substitutions without any negative impacts on AR functioning. However, in this domain, large deletions or aggregation of multiple variations are required for AR activity impairment. AR has indicated without any missense mutations in 5% of CAIS and 27% to 72% of PAIS patients (Ahmed et al., 2000; Melo et al., 2003; Galani et al., 2008; Ferlin et al., 2006).

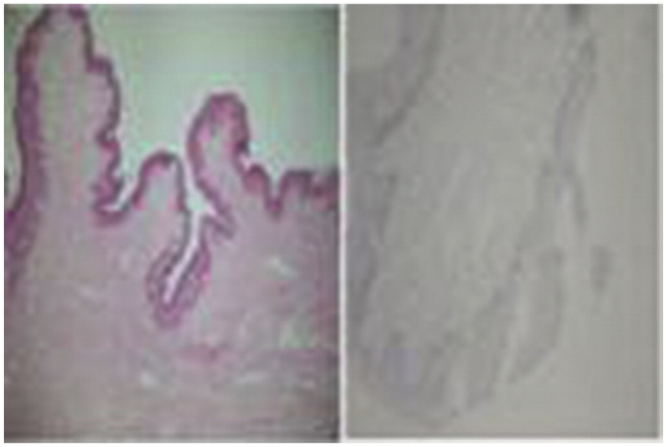

Fig. 3. Left: Histopathology features of skin tissue stained by H& E within unremarkable skin; Right: IHC staining of the same specimen for androgen receptor shows weakly positive result in IV1 and IV2 patients of family 1.

The presence of same phenotype in both siblings of family 2 could propose that the genetic influence for CAIS is quite significant. It was shown that the presence of unknown co-activators is required for the transmission of expression signal from AF-1 to basal transcription machinery. The lack of these co-activators could lead to PAIS presentation despite the AR gene would be intact (Horwitz et al., 1996). Some of the co-activators interacting with AF-2 region were determined including p300, androgen receptor-associated protein, transcriptional intermediary factor 2 and full-steroid-receptor co-activator-1. Since the G639A is a synonymous variant, its probable association with mutations of unidentified AF-1 co-activators could explain such abnormal clinical phenotype. Further studies are needed to determine AR co-activators that interact with AF-1 to shed light on the pathophysiology of AIS among AR mutation free cases.

Genetic counseling plays a crucial role in management of X-linked disorders such as AIS. There are various approaches to AIS genetic counseling and related family members. Sex selection through preimplantation genetic diagnosis (PGD) or prenatal diagnosis (PND) could be recommended for families with unknown mutation. In cases with known mutation, the confirmation of diagnosis is possible via direct molecular analysis through PGD or PND.

It should be noted that the improper or late diagnosis may expose AIS patients to the development of gonadoblastoma tumors, prostate cancer — particularly in carriers of LBD alterations and problems in socio-sexual identity (Gottlieb et al., 2004). In the consideration of puberty virilization putative occurrence, gonadectomy should be performed prior teenage (Kohler et al., 2005).

5. Conclusion

Our findings in family 1 could be a further proof for the pathogenicity of the "c.2522G > A variant". The c.639G > A (rs6152) variant in two siblings in family 2 could not be confirmed as the cause of disease. We suggest that the R841H mutation of LBD domain of AR protein correlates with PAIS phenotype. On the basis of clinical examinations, different molecular methods are employed to confirm PAIS diagnosis. In IHC the antigenic property of protein is targeted by assay. In molecular genetic testing any delicate changes of protein structure correspondent to nucleotide changes can be recognize by a simple, non-invasive and more accurate method. Therefore, the mutation screening of a sub region of AR protein, the LBD domain, could be considered as a crucial step in suspicious PAIS subjects. Since the early diagnosis of PAIS in children with DSD is highly needed for appropriate choosing of rearing gender, such molecular analysis could prevent undesired outcomes and pave the way for genetic counseling. According to our personal experience, for grade III to V PAIS, the gender change toward female against chromosomal sexuality would be more reasonable.

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